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Remodeling by fibroblasts alters the rate-dependent mechanical properties of collagen



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ABSTRACT

The ways that fibroblasts remodel their environment are central to wound healing, development of musculoskeletal tissues, and progression of pathologies such as fibrosis. However, the changes that fibroblasts make to the material around them and the mechanical consequences of these changes have proven difficult to quantify, especially in realistic, viscoelastic three-dimensional culture environments, leaving a critical need for quantitative data. Here, we observed the mechanisms and quantified the mechanical effects of fibroblast remodeling in engineered tissue constructs (ETCs) comprised of reconstituted rat tail (type I) collagen and human fibroblast cells. To study the effects of remodeling on tissue mechanics, stress-relaxation tests were performed on ETCs cultured for 24, 48, and 72 h. ETCs were treated with deoxycholate and tested again to assess the ECM response. Viscoelastic relaxation spectra were obtained using the generalized Maxwell model. Cells exhibited viscoelastic damping at two finite time constants over which the ECM showed little damping, approximately 0.2 s and 10–30 s. Different finite time constants in the range of 1–7000 s were attributed to ECM relaxation. Cells remodeled the ECM to produce a relaxation time constant on the order of 7000 s, and to merge relaxation finite time constants in the 0.5–2 s range into a single time content in the 1 s range. Results shed light on hierarchical deformation mechanisms in tissues, and on pathologies related to collagen relaxation such as diastolic dysfunction.

Statement of Significance

As fibroblasts proliferate within and remodel a tissue, they change the tissue mechanically. Quantifying these changes is critical for understanding wound healing and the development of pathologies such as cardiac fibrosis. Here, we characterize for the first time the spectrum of viscoelastic (rate-dependent) changes arising from the remodeling of reconstituted collagen by fibroblasts. The method also provides estimates of the viscoelastic spectra of fibroblasts within a three-dimensional culture environment. Results are of particular interest because of the ways that fibroblasts alter the mechanical response of collagen at loading frequencies associated with cardiac contraction in humans.

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1. Introduction

Interactions between living cells and their mechanical environments are central to a host of physiologic and pathologic events ranging from development to metastasis to wound healing. Fibroblasts remodel their extracellular neighborhood considerably when

activated, stiffening and compressing their extracellular matrix (ECM) environment [1–9]. During remodeling, fibroblasts secrete ECM proteins, including collagens, proteoglycans, glycoproteins, and proteases, and cross-linking proteins and enzymes [1–4,10]. Fibroblasts exert traction on the ECM and each other [9,11–13], and secrete soluble factors that affect neighboring cells and tissues in a paracrine manner [14]. This remodeling changes the tissue as a whole by establishing a network to link and organize individual cells [15–20].

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Although several studies have estimated the effects of this remodeling on ECM elasticity [5,21], much less is known about the viscoelastic effects of ECM remodeling. Fibroblasts are known to affect viscoelastic tissue relaxation in pathologies such as congestive heart failure. However, these effects have not been quantified, and there is a pressing need for data on viscoelastic remodeling of tissues.

Additionally, the cells themselves change during remodeling. Cytoskeletal disposition is known to change in response to perturbations in mechanical loading [22–24]. During wound healing, cytoskeletal structure is regulated to develop force against the ECM to close the wound [25]. Cell transformation and tumorigenicity are associated with a decrease in cell viscosity and elasticity [26]. Although well established techniques exist for estimating cell elasticity within engineered tissues [27,5], protocols are still needed to acquire information about cell viscoelasticity.

Engineered tissue constructs (ETCs) comprised of reconstituted rat tail (type I) collagen and human dermal fibroblast cells serve as *in vitro* models of this remodeling, and provide simplified systems in which to assess how remodeling affects the mechanics of cells and ECM [28,29]. These systems have been applied to study both linear and nonlinear elasticity of cells and ECM, but variations of time-dependent tissue mechanics by ECM remodeling has not been well characterized quantitatively.

The goal of this study was to establish the ways that fibroblast cells modulate their ECM viscoelastically and contribute to ETC-level viscoelasticity over the course of ETC remodeling. We studied these effects by performing viscoelastic relaxation tests on ETCs at three different timepoints. Following techniques that are standard for evaluation of ECM elasticity, ECM viscoelasticity was evaluated by treating ETCs with deoxycholate, a mild detergent which dissolves cell membranes and disperses cytoplasmic structures including the cytoskeleton. Tests were interpreted using a discrete spectral generalized Maxwell approach [30], which yields both elastic moduli and viscoelastic relaxation finite time constants. Results showed that cells actively adapted the ECM, and that cells relaxed at multiple timescales, including one that is fast compared to those of the ECM.

2. Materials and methods

2.1. Engineered tissue construct (ETC) preparation

ETCs were synthesized using procedures described in detail elsewhere [25]. Briefly, human dermal fibroblasts (Lonza, Allendale, NJ, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) at 37 °C and 5% CO₂. The media were changed every 3–4 days and the cells were split when cell confluency reached >80% of the dish surface. The cells were used for culturing ETCs at the 7th–10th passage. 0.5 million cells were mixed with 1 ml of a solution consisting of DMEM and 0.5 mg/ml type I rat tail collagen (harvested in our laboratory). The pH of this mixture was brought to neutrality using 0.2 M NaOH. 0.5 ml of this mixture was poured into hollow, cylindrical Teflon molds; the molds contained a central rod to create an annular well with outer and inner diameters of 14.9 mm and 9.5 mm, respectively. The final mixture was incubated at 37 °C with 5% CO₂ for 30 min to allow the collagen to polymerize. Then, the molds were filled with DMEM supplemented with 5% fetal bovine serum (FBS) and were kept in an incubator for 24, 48 or 72 h to allow the cells to remodel the collagen. Three specimens were tested at each of the three remodeling times, a total of nine specimens.

2.2. Stress-relaxation testing apparatus and protocol

Stress-relaxation tests were performed on the ring-shaped ETCs. ETCs were mounted within glass organ baths filled with

HEPES-buffered DMEM (pH 7.4) and 5% FBS, and kept at 37 °C, conditions standard for culturing engineered tissues [31]. One end was attached to an actuator connected to a stepper motor, and the other to a force transducer, as described elsewhere [25]. Tissues were allowed one hour to accommodate to the new media before the stress-relaxation test. The protocol started with 10 min of force monitoring to establish a baseline. This was followed by tissue preconditioning consisting of 5 sequential cycles of a 20% axial stretch, followed by a 30 min recovery interval. A preconditioning protocol is standard in testing of collagenous tissues [32]. Using a strain rate analogous to that of the subsequent characterization experiments and a strain magnitude twice that used in the characterization experiments yields repeatable results in the testing of ETCs [27,33].

In the characterization experiments, ETC rings were stretched 10% at 20%/s then held isometrically for 3600 s while force was recorded at 50 Hz. Nominal stress data were inferred from force data by dividing the force by the cross-sectional area measured for each specimen at the conclusion of the test, as described below. Note that, despite the preconditioning, the specimens likely experienced some permanent deformation over the course of the loading; by considering a transversely isotropic specimen with a Poisson ratio of 0.5 locked into its deformed configuration, the difference between the actual first Piola Kirchhoff stress and that we report can be estimated to be less than 10%. The strain amplitude of 10% strain was chosen because it represents the upper end of the linear range for a tendon [34]. Achieving this over 0.5 s is representative of strains in response to a brisk walking cadence and to stretching by the cardiovascular system. The stretch rate and prolonged monitoring were furthermore suitable for characterizing the temporal range of physiological responses [30,35].

2.3. Deoxycholate treatment

The specimens were returned to their baseline configurations and allowed to recover for 30 min to prepare for testing the contribution of the remodeled ECM to viscoelastic behavior of the ring constructs. For this purpose, DMEM + HEPES was replaced with 0.05% w/v deoxycholate in PBS (pH 7.4), and specimens were allowed to incubate for 60 min. The stretch-and-hold protocol was then repeated. Deoxycholate was chosen over inhibitors such as cytochalasin D and latrunculin because it enables lysis of cells without altering the mechanics of the protein structure in the remaining porous ECM [36,27].

2.4. Measurement of ETC dimensions

After the end of each experiment, specimens were mounted on spacers and stretched to their reference length, then fixed in 4% formaldehyde for 20 min at room temperature. Afterwards, specimens were cut into two equal pieces and placed within four-well plates filled with PBS. The width and thickness of each tissue were measured using Confocal microscopy (LSM 510, Zeiss). The thickness was measured near the upper and lower borders as well as in the middle of the tissue. The cross-sectional area of the tissue was calculated as the mean of the three measurements. All measurements were conducted by the same person.

2.5. DNA quantification

We synthesized an additional 9 specimens (three each at 24, 48, and 72 h of incubation) to estimate the final cell concentration in ETCs using a total DNA quantification assay. Right after the measurement of tissue dimensions, the constructs were centrifuged with 2 ml PBS in capped tubes. PBS was removed and, after 1 ml of lysis buffer was added, the tubes were sonicated. 30 µl of this sample was mixed with 3 ml of Hoechst solution (30 nM of

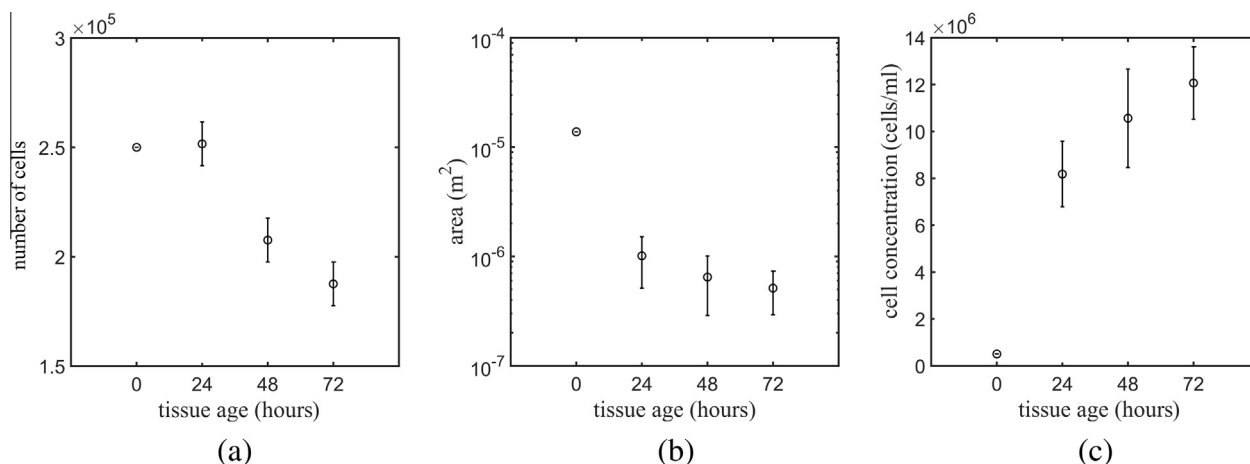


Fig. 1. Development of engineered tissue constructs (ETCs) over 72 h, including variations of their cross-sectional area, of the number of cells within them, and of the number of cells per unit volume (cell concentration). (a) The cross-sectional areas of the ETCs reduced to a minimum of $\sim 5 \times 10^{-7} m^2$ over approximately 36 h as the cells remodeled the collagen. (b) The cell population varied during this compaction, with the number of cells within each (initially 0.5 ml) ETC rising from 250×10^3 over the first 24 h of observation, then decreasing to approximately 190×10^3 over 72 h. (c) Throughout incubation, the cell concentration rose steadily. All error bars represent standard deviation.

Hoechst 33258 per ml of PBS) (Sigma, St. Louis, MO). The fluorescence of this mixture was read at excitation-emission of 346 nm–460 nm max, respectively. A known number of human dermal fibroblast cells was used as a calibration to estimate the number of cells in the mixtures.

2.6. Analysis of stress-relaxation data

The generalized Maxwell model was used to interpret the relaxation behavior of the specimens tested. We used a discrete spectral implementation described elsewhere [30], based on a large number ($n = 1000$) of exponential terms. In the discrete spectral approach, the relaxation times, τ_i , were logarithmically distributed equidistantly over a range broader than probable range of time constants of a material. The best fit elastic moduli E_i for each pre-selected τ_i were estimated by minimizing the mean squared error between the experimental data and the fitting arising from the estimated viscoelastic spectrum. We note that MSE is superior to R^2 for identifying viscoelastic relaxation spectra from relaxation spectra because even a mediocre fit can yield $R^2 \approx 1$ [30]. The spectrum arising by plotting E_i versus the pre-selected τ_i yielded an unbiased relaxation spectrum that enabled simple, graphical material identification.

2.7. Staining and imaging

The actin cytoskeletons of the fixed tissues were stained with rhodamine phalloidin (Sigma, St. Louis, MO) and imaged using confocal microscopy. Images were obtained with a Zeiss 510 confocal microscope using a $40\times$, 1.2 NA, water objective. In backscattering reflectance mode confocal microscopy, collagen fibers of ETCs can be observed directly without staining and with good axial and lateral resolution.

2.8. Statistical methods

All experiments were repeated in at least triplicate. Mean and standard deviation were reported. For estimates of the number of cells, cross-sectional area, and cell concentration in an ETC, mean and standard deviation were reported. Error bars were drawn to represent standard deviation. A key factor was the evolution of

morphology over time (specifically, the 24, 48 and 72 h time points). Students *T*-test was used to compare data at different time points, with $p < 0.05$ taken as the threshold for statistical significance. To show their statistical variance relaxation data and spectra, from all replicate tests were included in the [Supplementary document](#).

3. Results

3.1. Remodeling of ETCs

Over three days of remodeling, cells proliferated and compressed the ETCs (Fig. 1a), with the initial cross-section area (thickness \times width) decreasing from $13.8 \times 10^{-6} m^2$ (initial solution poured into the mold) to $1.01 \times 10^{-6} m^2$ during the first 24 h, and to $0.51 \times 10^{-6} m^2$ after 72 h. The starting population of 250×10^3 cells (in 0.5 ml of solution) was sustained for 24 h, and decreased to about 190×10^3 after 72 h (Fig. 1b). The initial cell concentration for all ETCs was 0.5 million cells/ml. Over 24, 48 and 72 h of incubation, cell concentration rose to approximately 8, 10 and 12 million cells/ml, respectively (Fig. 1c).

3.2. Mechanical responses

In response to the ramped stretch, the stress needed to maintain an ETC at its length increased to a peak value. As the ETC was held isometrically, this stress dropped over time as a result of viscoelastic relaxation for both the control (Fig. 2a, c, and e) and deoxycholate-treated (Fig. 2b, d and f) ETCs.

These stress-relaxation curves changed as the course of remodeling progressed, and were analyzed to quantify the effects of remodeling. The peak stresses attained immediately following cessation of the ramp tended to increase as the cells continued remodeling the ETCs over time for both the control and deoxycholate-treated ETCs. From the 24 to the 72 h time points, peak stress doubled for the control ETCs (Fig. 3a) and more than doubled for the deoxycholate-treated ETCs (Fig. 3b); these trends were significant statistically ($p < 0.001$). Additionally, the peak force in the ETCs rose significantly between 24 and 48 h, indicating that the stiffening extended beyond that associated with syneresis

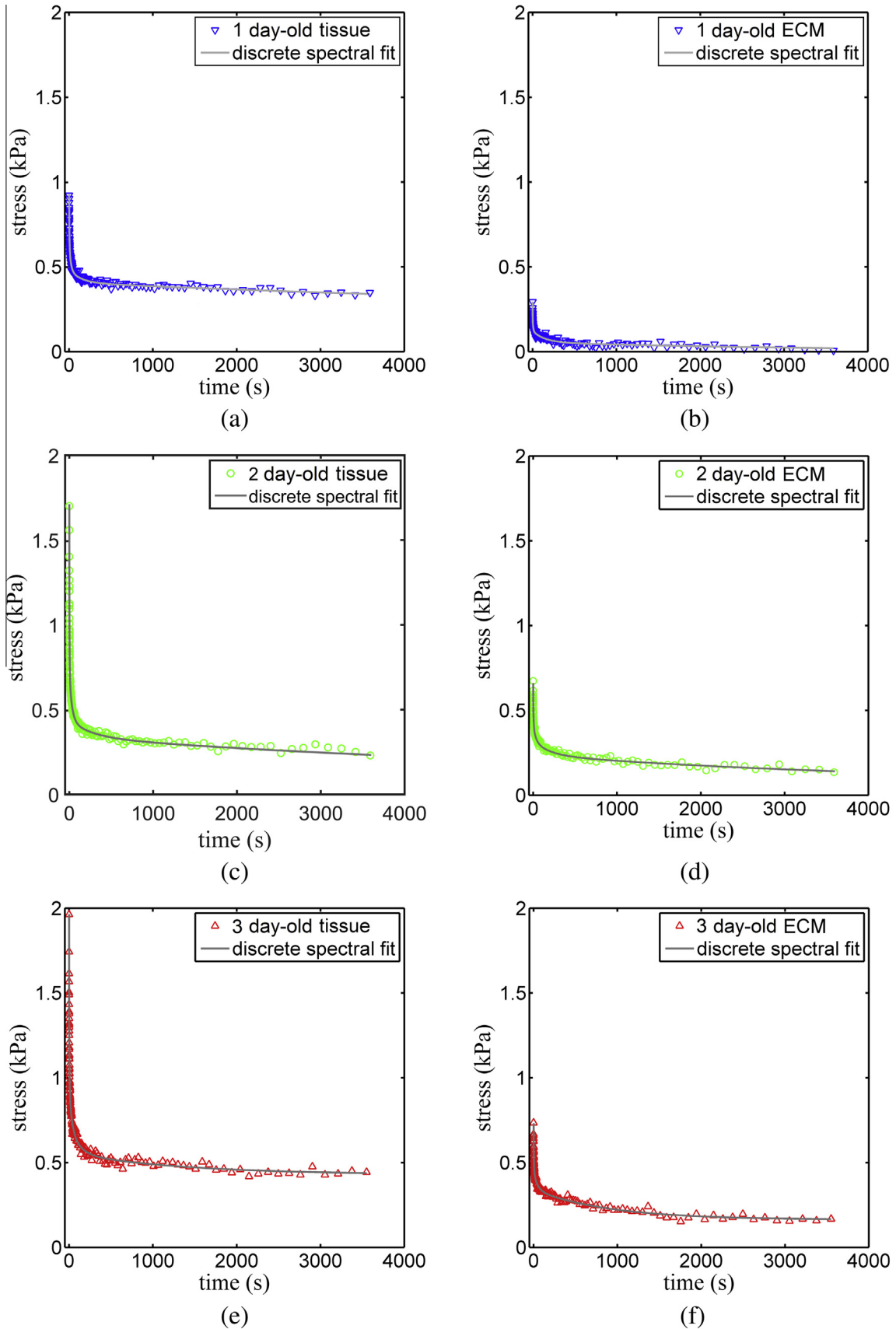


Fig. 2. Stress relaxation curves showing the variation of stress over time in engineered tissue constructs (ETCs) that were held isometrically at 10% stretch immediately following a linear ramp to 10% stretch over 0.5 s. The isometric force required to sustain ETCs at 10% stretch relaxed viscoelastically. Left column (a, c, e): ETCs tested in nutritional medium; right column (b, d, f): ETCs tested in nutritional medium plus deoxycholate to lyse cells. Three time points were considered: 24 h (a, b), 48 h (c, d), and 72 h (e, f). The data presented are representative; replicate data are presented in the [Supplementary document, Fig. S1](#) (24 h), [S3](#) (48 h), and [S5](#) (72 h).

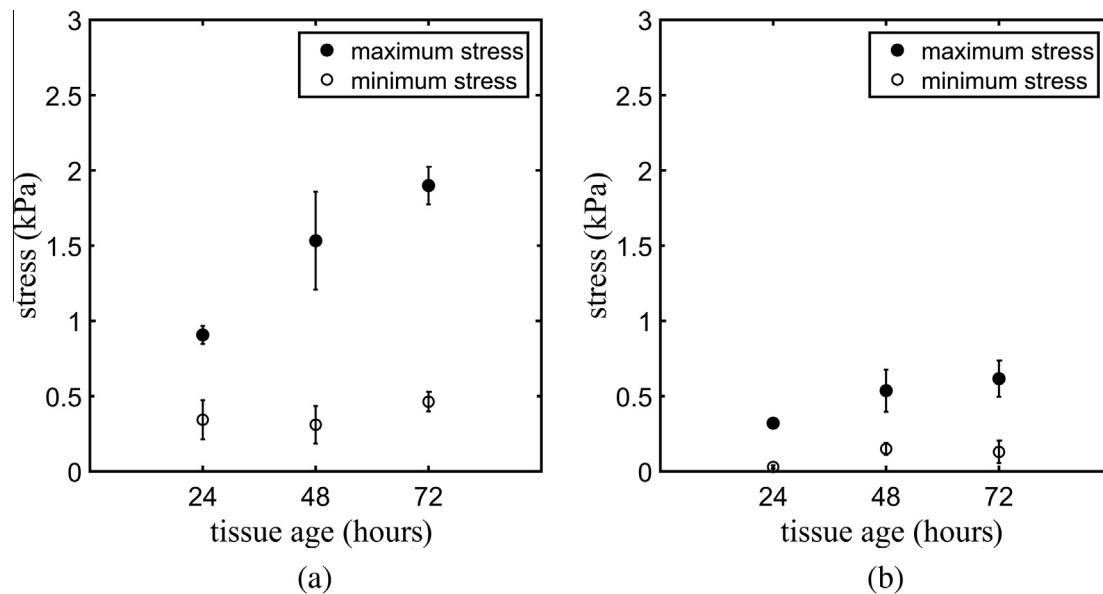


Fig. 3. Variations of the peak stress (immediately following ramp loading, solid circles) and minimum stress (reached after 3600 s of relaxation, hollow circles) attained during stress relaxation tests on engineered tissue constructs (ETCs) that remodeled for 24, 48, or 72 h prior to testing. (a) In ETCs tested in nutritional medium only, a statistically significant increase was observed in peak stress between 48 and 72 h. (b) In ETCs tested following treatment with deoxycholate, a significant increase was observed in the minimum stress between 24 and 48 h. Error bars: standard deviation. Criterion for significance: $p < 0.05$ in Student's *T*-test.

(Fig. S7). Treatment with dexamethasone more than halved the peak stress in each case. The minimum stress in the dexamethasone-treated ETCs rose from the 24- to the 48-h time point ($p < 0.05$, Fig. 3b).

The stress relaxation was analyzed using a spectral approach that, through integration with tests involving dexamethasone, quantified the rheological responses of the cells and ECM over a range of loading time constants. The analyses demonstrated that the relaxation mechanisms of ETCs cannot be described by one simple exponential term (Fig. 4).

The analysis showed subtle but significant transitions in some of the viscoelastic relaxation time constants over the course of ECM remodeling, with a slower finite time constant (~ 7000 s) emerging between 24 and 48 h of remodeling, and with some faster time constants (0.5–2 s) merging into a single time constant between 48 and 72 h of remodeling. As discussed below, these changes were retained following dexamethasone treatment, indicating that the changes occurred in the ECM rather than in the cells. In the dexamethasone treated ETCs, with cells eliminated, the 0.2 s time constant disappeared at all time points. At 24 h, the ~ 8 s time constant disappeared with dexamethasone treatment, and at 48 and 72 h, the 30 s time constant disappeared (Fig. 4b, d and f). The elastic moduli decreased for the dexamethasone treated ETCs with respect to control samples (Fig. 4a, c and e) at finite time constants faster than 100 s. Variance amongst the relaxation spectra was greatest for the 48-h time point.

3.2.1. Imaging of ETCs

Imaging of 24-h-old ETCs showed disordered collagen fibers (representative image, Fig. 5a). For 48-h-old ETCs, a transitional state could be observed in which collagen fibers were more organized and began to exhibit a preferred orientation (representative image, Fig. 5b). Finally, a very compact pattern of collagen fibers appeared in 72-h-old ETCs (representative image, Fig. 5c). This compact pattern could be found at a multitude of locations throughout the ETCs, not only close to the edges. Cells in ETCs could establish a network that linked and organized cells not only in the direction of circumferential restraint but also perpendicular to it (Fig. 6). The ECM contained several clusters of adjacent cells forming ring-like structures (e.g., Fig. 6).

4. Discussion

4.1. Cells remodeled the ECM locally

Many consequences of the interactions between cells and ECM were evident by observing the ETCs. Reflectance mode confocal microscopy, in which collagen fibers of ETCs can be observed directly without staining and with good axial and lateral resolution, showed that collagen fibers remodeled over time into a network showing elements of structural organization (Fig. 5). The latter typically aligned with the direction of circumferential constraint in the ring-shaped ETCs, and also connected cells or groups of cells (Fig. 6). Cells reduced ETC cross-sectional area as they collectively remodeled the ECM (Fig. 1).

Our images (Figs. 5 and 6) and data (Fig. S7, Supplementary document) were consistent with previous experimental results [37,15,16] showing that remodeling involves more than simple syneresis. Remodeling mechanisms are believed to include increasing cross-linking of the collagen network, which helps collagen retain a compressed state and increases collagen fibril stiffness [38,39]. Additionally, contractility [15,16], enzymatic activity [40], and matrix synthesis [17] play roles.

The structural aspects of remodeling are central to the mechanics of ETCs [13,31], and are particularly important for promising new therapies to improve cardiac function after myocardial infarction through guiding scar development [41]. They are also key to understanding how myofibroblasts invade an infarct region [42,43]. Additionally, these factors are central to healing of tendons, ligaments, and their bony attachments [44–50,35]. In all of these, percolation phenomena involving formation of a continuous structural network are important, and variance is typically highest in conditions close to percolation [44]. Indeed, in our study, the variance in relaxation spectra was highest in samples at the 48-h time point, when images showed the beginnings of interconnection amongst remodeled regions of ECM. Although visualizing and quantifying the underlying mesoscale effects are open challenges [51,52], an understanding of how viscoelasticity is mediated by such changes will be an important future component of such studies.

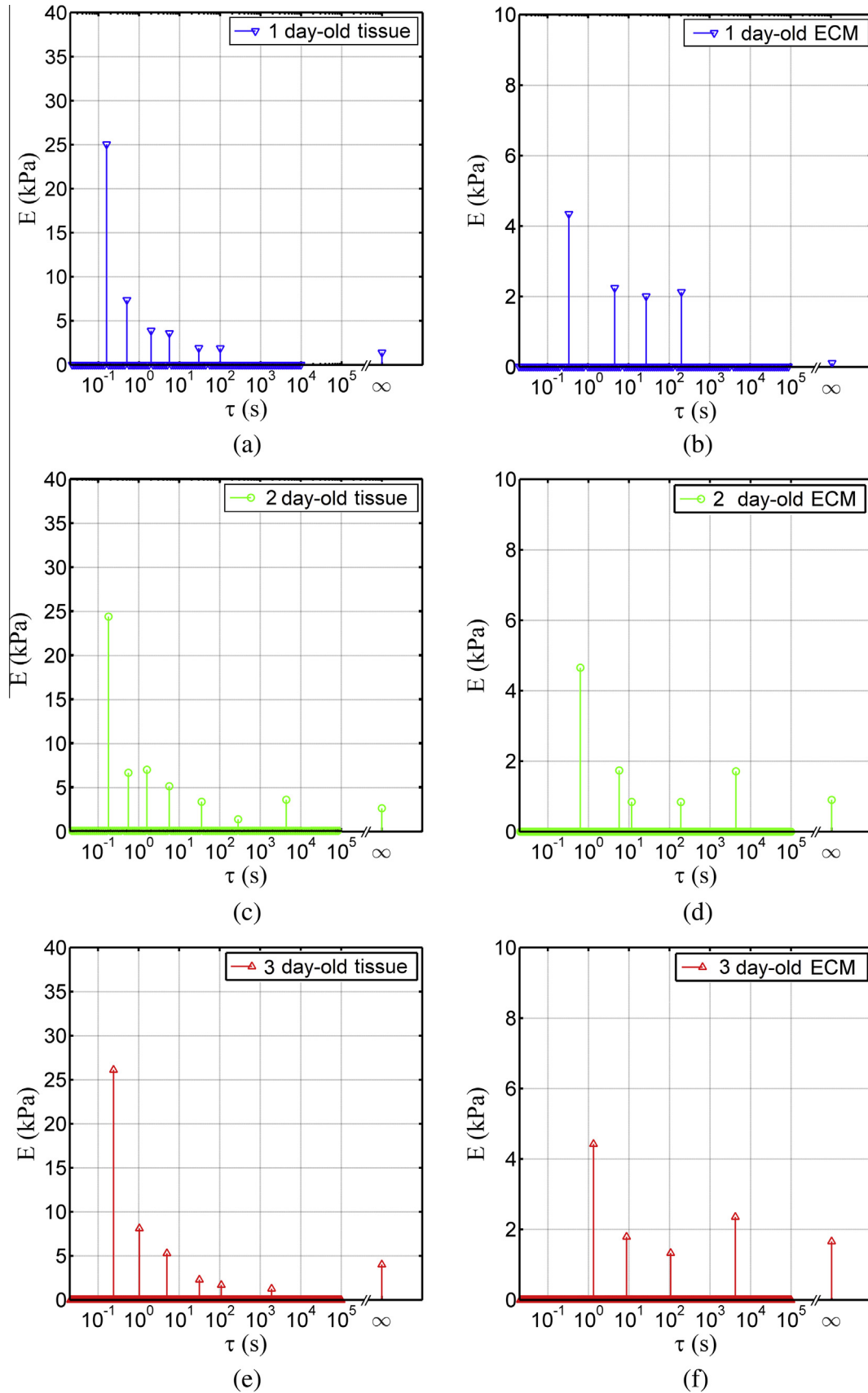


Fig. 4. Viscoelastic relaxation spectra of ETCs tested in nutritional medium (left column, panels a, c, and e) or nutritional medium plus deoxycholate to lyse cells (right column, panels b, d, and f). Cells and ECM displayed distinguishable contributions to the ETC viscoelasticity, with the latter changing over the course of ECM remodeling. Three time points were considered: 24 h (a, b), 48 h (c, d), and 72 h (e, f). The time constants of the ETCs were similar at the three different tissue ages. The exception was that during 48–72 h of the remodeling the two time constants between 1 and 10 s merged into a single time constant at about 2 s. In the deoxycholate treated ETCs the 0.2 s and 30 s peaks were absent. Each panel corresponds to a single, representative specimen. Replicate spectra are presented in the [Supplementary document, Fig. S2](#) (24 h), [S4](#) (48 h), and [S6](#) (72 h).

4.2. ETC remodeling occurred over specific ranges of relaxation time constants

A remarkable feature of the remodeling process was that the majority of the ETC relaxation time constants were *not* altered significantly (Fig. 4). Many others have quantified tremendous stiffening of ECM by cells [53–55] and substantial effects on the cells themselves [56], which appear to become more compliant with ECM stiffening in 3D [37] and more compliant in 2D [57]. Stiffening is typically small in tissue constructs that begin with cell concentrations below those in our study [58,5]. However, measurable contributions of cells to ETC mechanics are evident even at cell concentrations far below those used in the current study [59]. A number of recent studies have shown that, when cell density is sufficiently high, cells can adapt the fibrous structure of the ECM to form connections and structures like those shown in Figs. 5 and 6 [60,16,61–63,15].

In the work reported here, remodeling of ETCs by fibroblasts showed a major effect on peak stresses in stress-relaxation tests (Figs. 2 and 3). In this light, the relative lack of viscoelastic effects comes as a surprise. The only exceptions were that between 24 and 48 h, a relatively long finite time constant emerged in both the ETCs and the ECM, and that between 48 and 72 h of remodeling, two time constants 0.5 and 2 s merged into a single, intermediate time constant with a stronger amplitude. Because relaxation spectra are correlated to discrete structure of collagenous tissues [64–66], the merging of the two peaks indicates a decrease in the structural heterogeneity of the tissue during the remodeling process. These time constants are near those that others have been attributed to intra-fibril relaxation (~ 4 –7 s) [66,65,67]. One possible source of this merging of time constants is syneresis of inter-molecular water [68,69]. Time constants in the 1 s range are of particular interest for the cardiovascular system because this is associated with frequency of the heart. In fibrotic cardiomyopathy, a disease characterized by remodeling of heart tissue by activated cardiac myofibroblasts, a central pathology involves retarded diastolic filling [70,71], possibly due in part to viscoelastic remodeling. Although the fibroblasts within the heart are believed to differ in subtle ways from those in the skin, viscoelastic remodeling in this frequency range warrants further study.

4.3. Specific time constants can be attributed to cells, ECM, and components of the ECM

Responses of the tissue constructs to mechanical stretching were quantified using a spectral approach that, through integration with tests involving specific inhibitors, quantified the rheological responses of the cells and extracellular matrix (ECM) over a range of loading time constants. Through these tests, some responses of cells and remodeled ECM could be dissected. We note that the analysis techniques are robust against data that have not reached a complete steady state [30]. A time constant estimated by the discrete spectral approach can be trusted if it is infinite or if it is less than twice the duration of the viscoelastic relaxation data: error was found to be on the order of 7% for a time constant that is twice the duration of the viscoelastic relaxation data [30]. In Fig. 4, the slowest finite time constant was about 7000 s, which is within this trusted range.

The decrease in the minimum stress of the deoxycholate-treated with respect to non-treated (control) ETCs showed that at least a part of the time-independent (elastic) behavior of tissues, which corresponds to the single spring in the generalized Maxwell model, is due to elastic contributions of the cells. Other studies confirm that the cells have elastic (time-independent) properties [72,73].

Because deoxycholate treatment removed cells from ETCs, comparison of control and deoxycholate-treated ETCs enabled attribution of specific time constants to cell responses and to effects of ECM remodeling (Fig. 4). Six peaks in the relaxation spectra of 24 and 72-h-old ETCs demonstrated six relaxation mechanisms, while for the 48-h-old ETCs this number increased to seven. Two of the spectral peaks disappeared following treatment with deoxycholate (those at 0.2 s and 30 s for 48 h and older specimens; 0.2 s and 8 s for 24-h-old specimens) indicating that these are attributable to cells or cell-ECM interactions. Although cells might be expected to exhibit different relaxation behaviors under different environmental conditions, our results for the time constants of cells were within the range reported in other studies [72–74]. Wong et al. [75] reported that the major viscoelastic components of the cell cytoskeleton are responsible for vital mechanical functions, and found a slowest time constant for a cell of 0.28 s, a number within the range of our findings. Nekouzadeh et al. [12] and Treppe et al. [76] found that very fast relaxation was associated with cytoskeletal fluidization; although they did not quantify the relaxation spectrum, we note that this is one possible explanation for the fastest time constant we observed. We note as well that the elastic moduli decreased for deoxycholate-treated ETCs for finite time constants below 100 s (Fig. 4), possibly due to loss of fibroblast contractility and contributions of voids in the ECM left by the lysis of cells.

The remaining four time constants (five after 48 h) were all associated at least in part with the ECM. These time constants included several within the range of those found by others. Pryse et al. [77] found three plus an infinite time constant (approximately 5, 40, and 600 s); Xu et al. [67], who estimated a regularized, continuous spectrum, found dominant time constants between 0.3–1 s, 3–90 s and > 200 s for collagen matrices. We note that ETCs exhibited some faster relaxation time constants than did collagen matrices, likely due to the action of cells.

Much work in the literature has focused on identifying the hierarchical structural origins of the elastic (time-independent) and viscous (time-dependent) behaviors in collagenous tissues [78,79], including work on effects of microstructural changes on the microscopic viscoelastic properties of collagen at different hierarchical levels [80,68]. The idea is that by analyzing the stress-relaxation data, and by assuming that different time constants correspond to specific hierarchical structures, one can gain insight into hierarchical contributions and distinguish different materials [81–83]; the elastic modulus at each time constant of the relaxation spectrum reflects the contribution of that time constant to ETC relaxation. Potential sources of viscous relaxation exist across collagen's hierarchical organization [68,84]: triple-helix collagen molecules; cross-linked fibrils a few hundred nm in diameter assembled from these molecules in a roughly triclinic lattice, and infiltrated by water and non-collagenous proteins; and cross-linked fibers assembled from these fibrils.

The work of Gupta et al. [65] and Shen et al. [85] identify the 7–10 and 100–110 s time constants as being associated with intra-fibril relaxation, and 2 and 33 s time constants as being associated with inter-fiber relaxation. Gupta et al. [65] notes that no intra-fiber relaxation was evident in their experiments. Relaxation time constants for intra-fiber interactions have not yet been characterized in any study of which we are aware. The 7000 s time constant we observed might be associated with this, or might be a feature of the larger structures within the tissue construct. The relaxation time constants of the tropocollagen molecules are on the order of microseconds [68] and appear to be masked at the level of tissue constructs.

An additional factor is water flow. At the molecular level, tropocollagen molecules bond covalently [68] and are surrounded by a hydration layer [86]. At the next hierarchical level, water plays a structural role, possibly maintaining spacing between collagen

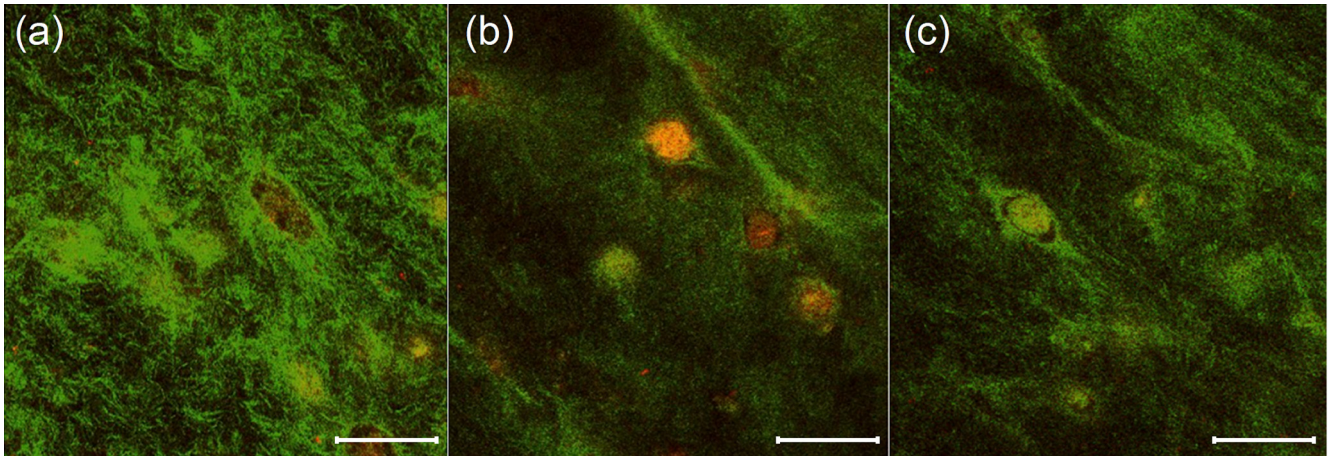


Fig. 5. Confocal reflectance microscopy images of collagen fibers (green) superimposed upon images of cells (red) in engineered tissue constructs. Although little effect was evident in the viscoelastic time constants, remodeling corresponded to a substantial change in ECM morphology. (a) A random collagen distribution was evident at 24 h. (b) The collagen fibers were more organized and began to exhibit a preferred orientation at 48 h. (c) A very compact pattern of collagen fibers was observed at 72 h. Red: cell membranes; green: signal from confocal reflectance. Scale bar, 50 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

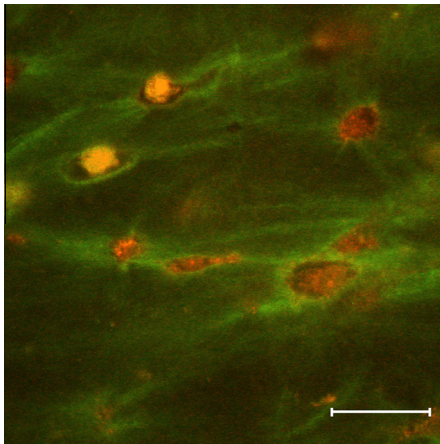


Fig. 6. A confocal reflectance microscopy image of collagen fibers (green) superimposed upon images of cells (red) in engineered tissue constructs. During the remodeling, the cells established a connective network, with some clusters of adjacent cells forming ring-like structures. The network appeared to link and organize individual cells in the ECM. Mechanical signals sent to amongst cells over a timescale faster than the fastest time constant in these collagen bundles are not attenuated by the bundles. Scale bar, 50 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fibrils, while dehydration causes the tighter packing of fibrils and enhances mechanical rigidity by increasing intra-molecular hydrogen bonds [87–89]. Gautieri et al. [90] found that the dominant fibril-level deformation mechanism shifted from molecular sliding to molecular stretching with dehydration, with the latter exhibit higher stiffness.

Debate exists in the literature about how cross-linking affects stress-relaxation. Xu et al. [67] concluded that the viscoelastic response of a collagen gel depends upon cross-linking, while Feng et al. [91], comparing viscosity of artificial and native tissues, concluded that it does not. From the observations of remodeling dependent viscoelastic responses in this article, which includes effects of cross-linking, one can speculate that both groups are correct. The 100–110 s intra-fibril time constant persists throughout remodeling, while the 7–10 s time constant associated with intra-fibril relaxation seems coupled with cell responses in ETCs tested at 24 h.

4.4. Caveats

A limitation of the current work is that the data acquired are strictly uniaxial. The off-axis contributions of cells to tissue viscoelasticity cannot be quantified. Previous data show that under the conditions of these experiments, the additive decomposition of the contributions of cells and ECM is a reasonable approximation [7,6]. Moreover, for elongated cells appearing in these tissue constructs, the contributors of the transverse behavior of cells to the mechanics of the tissue construct is on the order of only a few percent [92].

A question arising in the study and elastic analysis of ETC mechanics is whether the mechanics of the porous ECM arising from deoxycholate treatment is representative of the true ECM mechanics. Although this issue is largely resolved in the elastic analysis of tissue constructs [27], the question bears scrutiny in our viscoelastic analyses. Two questions arise. First, does deformation of pores in which cells resided an important (and irrelevant) deformation mechanism? If this were the case, then a new time constant or set of time constants associated with such deformation would be expected to appear in the relaxation spectrum. However, no such time constants appeared, and time constants associated with cells or cell/ECM interactions disappeared. Second, although deoxycholate does not change the short-term or long-term elastic responses of the ECM [5], does it change the viscoelastic response? Again, neither substantial shifting of time constants nor appearance of new time constants was observed in the ECM relaxation spectra. Although the possibility exists that some of the time constants attributed to cells disappeared because of the action of deoxycholate, the absence of elastic effects combined with the overall insensitivity of the remaining time constants makes this possibility appear remote. However, now that tools for doing so are becoming available, quantifying the viscoelastic effects of deoxycholate in addition to the other agonists, inhibitors, and detergents that are applied commonly in the study of ETCs represents an important direction of future inquiry.

5. Conclusions

Although the hierarchical origins of the time constants observed are still an issue of debate, the spectral analyses we presented indicate that cells remodel the viscoelastic nature of their

environment, and have the potential to communicate through a viscoelastic ECM at higher frequencies. Our spectral analyses revealed that human dermal fibroblasts remodeled their environments in such a way as to strengthen some temporal responses relative to others. These changes resulted in an increase in the energy absorption in the 1 Hz range, indicating increased damping in the range associated with heart contraction in humans. The cells themselves exhibited damping at time constants sufficiently fast that the ECM would effectively appear elastic, indicating that cells are capable of absorbing and transmitting mechanical signals from one another at these ranges. Results highlight the multifaceted nature of the signals that cells receive, and indicate that the well-known ECM stiffening effects of fibroblasts are enacted over a specific range of viscoelastic time constants. The possible effects of this on cell–cell communication and pathologies of fibrosis warrant continued study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2016.03.034>.

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